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A novel subunit of axonemal dynein conserved among lower and higher eukaryotes

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Abstract To elucidate the subunit composition of axonemal inner-arm dynein, we examined a 38 kDa protein (p38) co-purified with a *Chlamydomonas* inner arm subspecies, dynein d. We found it is a novel protein conserved among a variety of organisms with motile cilia and flagella. Immunoprecipitation using specific antibody verified its association with a heavy chain, actin and a previously identified light chain (p28). Unexpectedly, mutant axonemes lacking dynein d and other dyneins retained reduced amounts of p38. This finding suggests that p38 is involved in the docking of dynein d to specific loci.

Keywords: Inner arm dynein; Light chain; p28; Actin; *Chlamydomonas*

1. Introduction

Cilia and flagella contain distinct types of dyneins that differ in function and subunit organization (see [1] for review). In *Chlamydomonas*, a model organism for cilia/flagella studies, axonemal dyneins can be classified into three types: an outer arm dynein with three heavy chains; an inner arm dynein with two heavy chains; and at least six species of inner arm dynein with single heavy chains. The last type of dynein (one-headed type) contains actin and either centrin or a 28 kDa protein (p28) beside the heavy chain [2,3]. These proteins have been found in various organisms with motile cilia and flagella, such as sea urchin [4], mouse [5] and humans [6], indicating a conserved design.

However, possibly there may be other subunits of one-headed inner arm dyneins. We previously found that chromatographically isolated dynein subspecies d (referred to as dynein d), a one-headed dynein, contained a 38 kDa and a 44 kDa protein. They were missing in the corresponding fraction from *ida4*, a mutant lacking dynein d and two other dyneins [3] because of a mutation in the p28 gene [7]. Here, we examined the 38 kDa protein (p38) by cloning its cDNA and producing specific antibody. Our data show that p38 is in fact a component of dynein d. Homologues of this protein are present in a variety of organisms with motile cilia and flagella, suggesting that dynein d is also conserved and possibly performs a unique function in cilia and flagella motility.

2. Materials and methods

2.1. Strains and culture

Chlamydomonas reinhardtii strains 137c (wild type; wt), *oda1* (lacking outer arm dynein), *ida3* (lacking inner arm dynein f), *ida4* (lacking inner arm dynein a, c and d), *ida5* (lacking inner arm dynein a, c, d and e), and *ida6* (lacking inner arm dynein e) [1] were used. Cells were grown in Tris–acetic acid-phosphate (TAP) medium with aeration.

2.2. Crude dynein extract and isolation of dynein

Flagella were isolated and demembranated with 0.2% Nonidet P-40 in HMDEK solution (30 mM HEPES, 5 mM $MgSO_4$, 1 mM DTT, 1 mM EGTA, and 50 mM potassium acetate, pH 7.4) [8]. Inner arm dyneins were extracted from the axoneme with 0.6 M KCl in HMDEK [9], and fractionated into individual dynein species by ion-exchange chromatography [3]. To achieve better separation of dynein d, we used a Uno-Q column (Bio-Rad) instead of a Mono-Q column.

2.3. Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was carried out using a linear 5–20% sucrose density gradient prepared in HMDEK solution containing 0.2 mM protease inhibitor (Pefa Bloc). The gradients were centrifuged in a Hitachi RPS55T-2 rotor at $180\,000 \times g$ (44 000 rpm) for 5.5 h at 4 °C. Thirteen fractions (400 μl each) were collected.

2.4. Protein identification

The 38 kDa band in the SDS–PAGE patterns of axonemes was excised, digested with trypsin, and analyzed using a oMALDI-Qq-TOF MS/MS QSTAR Pulsar *i* (Applied Biosystems). The obtained data were then used to search the genome database (JGI v. 2.0, http://genome.jgi-psf.org/chlre2/chlre2.home.html) with the algorithm Mascot (http://www.matrixscience.com/).

2.5. Bacterial expression of p38

The coding region of the p38 cDNA was amplified by PCR with primers P38-F (CTGGATCCGCGACCTTAACGTATACAGTGTTCTCC) and P38-R (GAGAATTCCCCGCCCGTCAGCGCG), which contained the recognition sites for *Bam*HI and *Eco*RI, respectively (underlined). The PCR product was ligated to the *Bam*HI and *Eco*RI sites of the bacterial expression vector pCold. The resulting fusion protein contained His- and GST-tag sequences at its N terminal. Expression of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to a logarithmically growing culture of *Escherichia coli*. Almost all of the expressed protein was contained in inclusion bodies. The inclusion bodies were purified and solubilized in 8 M urea.

2.6. Immunological analyses

The recombinant p38 protein was electrophoretically purified and used to immunize two rabbits. Antibodies obtained were affinity purified using recombinant p38 blotted on polyvinylidene difluoride membranes. Immunoblot analyses were carried out after the method

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of [10]. Immunoprecipitation was performed after [11] using the p38 antibody and protein A beads (Roche Diagnostics Corp.).

2.7. Immunofluorescence microscopy

Immunofluorescence microscopy was performed according to [12]. The nucleoflagellar apparatuses of *oda1* were isolated and fixed with 2% paraformaldehyde and cold acetone (−20 °C). Fixed samples were stained with the p38 antibody and fluorescein isothiocyanate-labeled anti-rabbit IgG antibody.

3. Results

3.1. Identification of p38

Inner arm dyneins in the crude extract from wild-type axoneme were fractionated by ion exchange chromatography into nearly single species. SDS–PAGE analysis confirmed the previous finding that the fraction of dynein d contains a 44 kDa and a 38 kDa band in addition to those of the two established subunits, actin and p28 (Fig. 1A) [3]. In this study, we examined the 38 kDa protein. First, we carried out mass spectrometry using isolated protein samples cut out from the gels and digested with trypsin. This resulted in identification of five partial amino acid sequences. Search in JGI database of *C. reinhardtii* (release 2.0) for these sequences revealed that this protein has been registered in the flagellar proteome database [13] as FAP146, a Conserved Uncharacterized Zinc Finger-Like Flagellar Associated Protein (JGI Linkout: Version 2: C_30068, Protein ID 164568).

The cDNA of p38/FAP146 is predicted to encode a 380-amino acid protein with a molecular weight of 40923 Da and a pI of 5.29. BLAST search identified putative homologues of unknown function in a wide range of ciliated organisms such as *Trypanosoma* (BLAST *E*-Value = 2 e−14), Zebrafish (3e−15), Mouse (2e−14) and Human (5e−16) (Fig. 2). No obvious homologues are found in organisms with only immotile axonemes, such as *C. elegans*. A MYND zinc-finger domain is present in the homologues of higher eukaryotes but not in *Chlamydomonas* or *Trypanosoma.*

3.2. P38 is diminished in the mutants lacking inner dynein arm d

A polyclonal antibody was raised and affinity-purified against bacterially produced p38 protein. Immunoblot analysis of axonemes demonstrated that the antibody recognized a single band corresponding to p38 (Fig. 1B). Unexpectedly, p38 was diminished but not completely absent in the axonemes of *ida4* and *ida5*, both of which lack dynein d and a few other inner arm dyneins (Fig. 1C and D). Comparison of the band densities in these axonemes with successively diluted wt axonemes led to an estimate that p38 in these mutants is reduced to 30–50% of the wild-type level.

3.3. Immunoprecipitation

To determine the proteins associated with p38, we carried out immunoprecipitation using anti-p38 antibody on the crude dynein extracts from wt and *oda1* axonemes. The resultant precipitate always contained, in addition to p38, a 42 kDa protein and a high-molecular weight protein of the size of a dynein heavy chain. The protein p28, a subunit of several types of inner arm dyneins including dynein d, was also precipitated as detected by a specific antibody (Fig. 3A and B). The 42 kDa protein is most likely actin, which is also a subunit of inner arm dyneins. To identify the precipitated heavy chain, we carried out mass spectrometry on the protein band cut out from the gel. Peptide mass finger print analysis revealed that the heavy chain is DHC2 dynein heavy chain (JGI Linkout: Version 2: C_80197, Protein ID 171127). In an independent series of experiments, we have recently identified the DHC genes of all known one-headed type inner-arm dyneins, and found that DHC2 corresponds to inner arm dynein d (Yagi and Kamiya, unpublished data). Therefore, these results indicate that p38 is associated with a complex that contains DHC2, actin, and p28; i.e., proteins that have been previously identified as the components of dynein d. The immunoprecipitation experiment did not determine whether the 44 kDa protein, another putative subunit of dynein d, is also contained in the precipitate, because the protein band was overlapped with the heavily stained

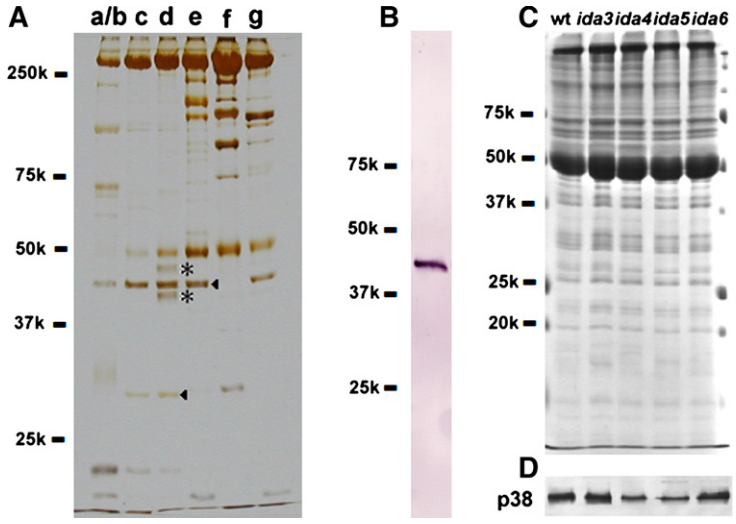

Fig. 1. Presence of p38 in dynein d and axoneme. (A) Fractionation of high-salt extract of wt axonemes by chromatography on a Uno Q column. SDS–PAGE patterns of the peak fractions of inner arm dyneins. An 11% gel stained with silver. The arrowheads indicate actin and p28. The asterisks indicate p44 and p38. (B) Western analysis of the wt axonemes with affinity purified p38 antibody. Only a single band of ~38 kDa is recognized. (C,D) Reduction of p38 in *ida4* and *ida5* axonemes as detected by Western analysis. (C) SDS–PAGE of the same amount of axonemes stained with CBB. (D) Same samples detected by immunoblot analysis with p38 antibody.

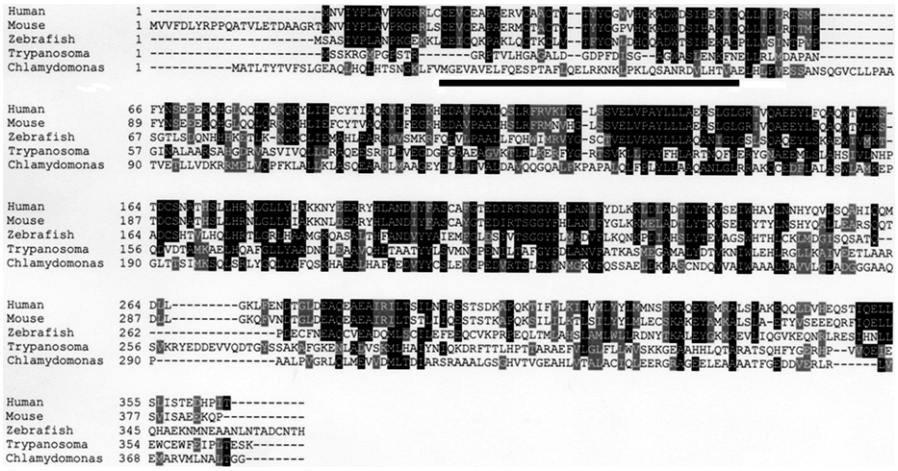

Fig. 2. Sequence analysis of p38. ClustalW alignment of the amino acid sequence of *Chlamydomonas* p38 with those of putative homologues in other organisms. Identical and conservatively substituted amino acids are shown with black and gray backgrounds, respectively. The black bar indicates a MYND zinc finger domain, of which the characteristic cysteine and histidine residues are absent in the corresponding sequence of *Trypanosoma* or *Chlamydomonas*.

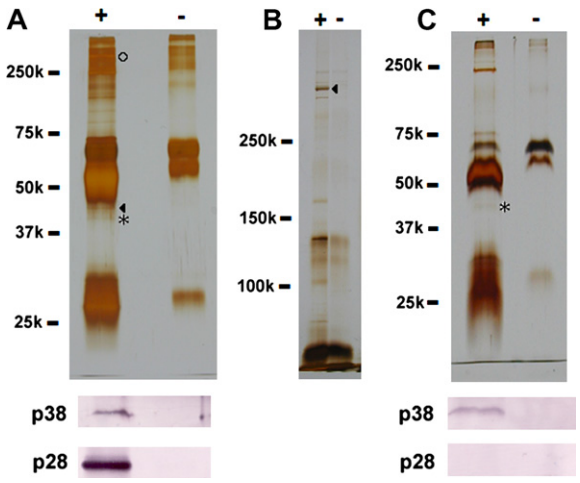

Fig. 3. Immunoprecipitation analysis using p38-specific antibody. (A) Immunoprecipitates from *oda1* axonemal extract. SDS–PAGE on an 11% gel. +, precipitates after incubation with p38 antibody and protein A beads. −, precipitates produced with protein A beads only. Dynein heavy chain (circle) and a 42-kDa protein (arrowhead) were precipitated in addition to p38 (asterisk). The 42-kDa protein is most likely actin, a subunit of inner dynein arm d. p28 was also precipitated. (B) SDS–PAGE (3–5% gel) of immunoprecipitates from *oda1* extract, showing precipitation of a dynein heavy chain (arrowhead). (C) SDS–PAGE pattern (11% gel) of the immunoprecipitate from the *ida5* extract. Neither dynein heavy chain nor actin is detected. Western blot showed that p28 does not precipitate either. Asterisk; p38. The identities of several co-precipitated proteins, including a 130 kDa protein seen in (B) and a 240 kDa protein seen in (C), are unknown.

IgG heavy chain band. Immunoprecipitation using the extract from *ida5* axonemes, which do not contain dynein d, did not yield dynein heavy chain, actin, or p28 in the precipitate (Fig. 3C). This result suggests that p38 in the *ida5* axoneme is not associated with p28 or actin.

3.4. Association of p38 with a dynein heavy chain in wild-type but not in ida5 axonemes

One-headed inner arm dyneins have been shown to sediment at 11–13 S in sucrose-density gradient centrifugation [2]. To see if p38 is present mostly associated with this type of dynein, high-salt axonemal extract was subjected to 5–20% sucrose gradient centrifugation. As shown in Fig. 4, the distribution pattern of the p38 protein in the wild type extract had a peak in the fractions where the one-headed dynein sediments; the peak of p38 distribution coincides with that of p28, a subunit of one-headed dyneins (Fig. 4B). Although the distribution is broad, no other peaks are present. The broad distribution may indicate that, in the high-salt extract, part of p38 is dissociated from the heavy chain and produces various aggregates with itself or with other proteins. In contrast, the high-salt extract from the *ida5* axoneme lacking dynein d had no peaks at 11–13 S but had one at ~3 S. This result suggests that, in the axonemal extracts of *ida5*, p38 is not associated with any dynein heavy chain.

3.5. Immunofluorescence localization

Indirect immunofluorescence microscopy on nucleoflagellar apparatuses demonstrated that p38 is uniformly localized

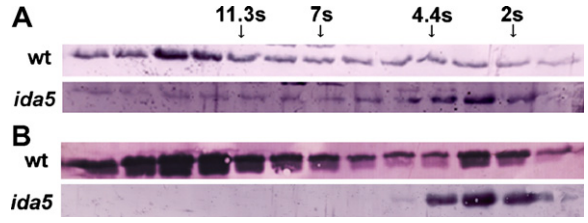

Fig. 4. Sucrose density gradient centrifugation analysis. Extracts from wt and *ida5* axonemes were fractionated on 5–20% sucrose gradients and probed with the p38 antibody (A) and p28 antibody (B) . In the wt extract, p38 sedimented at >11.3 S, in the same region where p28 sedimented, while it sedimented at ~3 S in the *ida5* extract. This indicates that p38 is attached to the one-headed dynein in the wild-type extract but not in the *ida5* extract. The distribution patterns of p28 in wild type and *ida5* axonemes suggest that p28 is also present in the *ida5* axoneme without associating with dynein heavy chains. A small fraction of p28 from the wild type axoneme appears to be dissociated from dynein heavy chains, as evidenced by the increased density at 3 S in (B). The arrows with numbers are the peak positions of catalase (11.3 S), aldolase (7 S), bovine serum alubumin (4.4 S) and ribonuclease A (2 S).

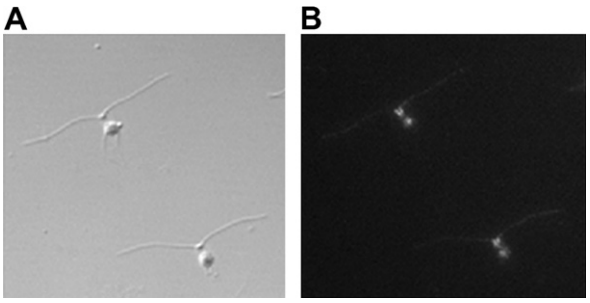

Fig. 5. Immunofluorescence localization of p38. (A) Differential interference contrast and (B) indirect immunofluorescence images of the nucleoflagellar apparatuses from *oda1*. Stained with the p38 antibody and an FITC-conjugated goat anti-rabbit IgG. Flagella and the basal body region are stained. Neither flagella nor basal body/nucleus was stained with the pre-immune serum and the secondary antibody (data not shown).

along the length of the axonemes and, more strongly in the basal body region (Fig. 5). The axonemal staining was usually greater in the *oda1* mutant lacking outer arm dynein than in wild type, probably because the bulky outer dynein arms in fixed wild-type samples blocked antibody access to the inner dynein arms.

4. Discussion

We have identified a 38 kDa protein (p38, FAP146) as a new subunit of inner arm species d. Until present, only actin, centrin and p28 have been known as the subunits of one-headed type inner arm dyneins [2]. Therefore, p38 is the fourth subunit. Importantly, BLAST search indicated that p38 has homologues in various organisms with motile cilia and flagella but not in organisms that have only immotile cilia. Together with the previous findings that p28, as well as actin and centrin, is preserved in organisms with motile cilia and flagella [4–6], our present findings suggest that the subunit composition of one-headed inner arm dyneins is preserved among organisms with motile axonemes.

Immuno-localization using specific antibody has shown that p38 is present in the basal body and nucleus, in addition to the axoneme. This indicates that some proteins in the basal body and nucleus share a common epitope with p38, the antibody we used is not specific enough for p38, or p38 is in fact present in the basal body/nucleus region. Further studies are necessary for identification of the immunoreactive protein(s) in loci where dynein is absent. The p38 localization on the axoneme was found to be fairly homogeneous along the length. Piperno and Ramanis [14], based on the analysis on the dynein composition in short flagella, proposed that some inner arm species are localized only at the base or tip of an axoneme. Our data suggest that the localization of at least dynein d is not restricted to the tip or basal region of the axoneme.

Unexpectedly, *ida4* and *ida5*, mutants lacking dynein d, were found to retain certain amounts of p38. Since p38 does not form an 11–13 S complex in the crude extract from the *ida5* axoneme, it must be present in the mutant axonemes without associating with dyneins. This observation led us to speculate that p38 constitutes the docking site for dynein d, because a protein capable of binding to the axoneme as well to a particular dynein fulfills the conditions required for such a docking protein. Previous studies have suggested that p28 and actin are involved in localization of several species of one-headed dyneins [3,15]. The protein p28 is directly associated with the tail portion of dynein heavy chain corresponding to the N-terminal region, as well as with actin [16]. The novel light chain p38 may also be associated, directly or indirectly, with the tail region of the N-terminal region of the dynein d heavy chain, although our chemical crosslink experiments have failed to detect proteins that interact with p38. Determination of the binding partner of p38 remains an important problem.

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